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Role in Virulence of a *Brucella abortus* Protein Exhibiting Lectin-Like Activity

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***Brucella abortus* is a facultative, intracellular zoonotic pathogen which can cause undulant fever in humans and abortions in cattle. A 14-kDa protein of *B. abortus* was previously identified to be immunogenic in animals infected with *Brucella* spp. In this study, we discovered that the 14-kDa protein possessed immunoglobulin binding and hemagglutination properties that appeared to be based on the protein's lectin-like properties. Hemagglutination inhibition experiments suggested that the 14-kDa protein has affinity towards mannose. Disruption of the gene encoding the 14-kDa protein in virulent *B. abortus* strain 2308 induced a rough-like phenotype with an altered smooth lipopolysaccharide (LPS) immunoblot profile and a significant reduction in the bacterium's ability to replicate in mouse spleens. However, the mutant strain was stably maintained in mouse spleens at 2.0 to 2.6 $\log_{10}$ CFU/spleen from day 1 to week 6 after intraperitoneal inoculation with 4.65 $\log_{10}$ CFU. In contrast to the case for the smooth virulent strain 2308, in the rough attenuated strain RB51 disruption of the 14-kDa protein's gene had no effect on the mouse clearance pattern. These findings indicate that the 14-kDa protein of *B. abortus* possesses lectin-like properties and is essential for the virulence of the species, probably because of its direct or indirect role in the synthesis of smooth LPS.**

The members of the *Brucella* genus are small, nonmotile, gram-negative, facultatively intracellular bacteria that can cause brucellosis in a variety of mammals. Brucellosis is a chronic zoonotic disease resulting in undulant fever in humans and abortion and/or infertility in affected animals. There are six species of *Brucella* that are currently recognized based on host specificity. They include *Brucella abortus* (cattle), *B. melitensis* (goats), *B. suis* (hogs), *B. canis* (dogs), *B. ovis* (sheep), and *B. neotomae* (wood rats) (7). Recently, *Brucella* has been isolated from a variety of marine mammals, including cetaceans (e.g., dolphins), seals, and otters (11).

Brucella organisms can be phenotypically categorized based on their colony morphology into smooth, rough, and intermediate/mucoid types (7). Organisms characterized as smooth contain the O antigen (O-polysaccharide composed of perosamine polymers) in their lipopolysaccharide (LPS); true rough organisms do not express the O antigen. In general, smooth *Brucella* species are more virulent than their rough counterparts. *B. canis* and *B. ovis* are the only species of *Brucella* that naturally occur in the rough form and yet are still pathogenic in their host species. All four other named species naturally occur in the smooth form; the newly discovered marine isolates all appear to be smooth.

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The basis for the virulence of *Brucella* species can be attributed to the ability of these bacteria to escape the host defense mechanisms and to survive and replicate within host cells. Virulent *Brucella* organisms are capable of invading and replicating in professional phagocytes (4) such as macrophages as well as in nonphagocytic cells (9, 10). The mechanism of attachment and entry into these cells by *Brucella* has yet to be clearly elucidated. However, using various mutagenesis techniques, several factors of *Brucella* necessary for host cell invasion and intracellular survival have been identified. These factors include smooth LPS, a type 4 secretion system encoded by the genes of the *virB* operon, the *bvrS-bvrR* two-component system, and cyclic β-1,2-glucan (13). Mutations in several other genes such as *purE*, *bacA*, and *hfq* have been shown to reduce the intracellular survival of *Brucella* (14).

Although antibodies to the O antigen can confer a certain level of protection against smooth *Brucella* infection, cell-mediated immunity (CMI) plays a major role in protection against brucellosis. Therefore, attenuated live vaccines such as *B. abortus* strains 19 and RB51 and *B. melitensis* Rev1, which induce strong CMI, are far superior to killed vaccines in conferring protection against brucellosis in target animal species (24). Several immunogenic proteins of *Brucella* have been identified and characterized (14). Appropriate immune responses generated against some of these proteins have been shown to confer protection in mouse models.

The initial objective of the present study was to examine the protective potential of a previously described 14-kDa immunoreactive protein of *B. abortus* that evoked an immune response in experimentally and naturally infected hosts (6). However, during this study, we discovered that this protein

TABLE 1. Bacterial strains and plasmids used for the present study

Strain or plasmid	Description	Reference or source
B. abortus 2308	Smooth, virulent strain	G. Schurig
B. abortus RB51	Rough, attenuated vaccine strain derived from *B. abortus* 2308	23
E. coli DH5α	Used for initial cloning and subcloning of the 14-kDa protein's gene sequences	Gibco BRL
E. coli BL21(DE3)	Lambda DE3 (*lacI lacUV5-T7* gene 1 *ind-1 sam-7 nin-5*), used for overexpression of the 14-kDa protein	Invitrogen
pBBR1MCS	Broad-host-range plasmid; capable of replication in *E. coli* and *Brucella*; confers chloramphenicol resistance	16
pBBGroE	pBBR1MCS derivative; contains *Brucella groE* promoter	28
pBBR4MCS	Broad-host-range plasmid; capable of replication in *E. coli* and *Brucella*; confers ampicillin resistance	15
pCR 2.1	3′-Thymidine nucleotide overhangs at insertion site for easy cloning of PCR products	Invitrogen
pRSET B	Plasmid for overexpression of cloned gene in *E. coli* in fusion with T7 and histidine tags; expression under T7 promoter	Invitrogen
pRSETssL14	A pRSET B plasmid containing the DNA sequences encoding the 14-kDa protein without the putative signal peptide; used for overexpression of the 14-kDa protein in *E. coli* BL21(DE3)	This study
pTAup14	A pCR2.1 plasmid containing the PCR-amplified fragment of the 14-kDa protein's gene along with its 5′ flanking sequences that include the putative promoter element	This study
pTARBS14	A pCR2.1 plasmid containing the PCR-amplified fragment of the 14-kDa protein's gene along with its 5′ flanking sequences that include the ribosomal binding site but not the putative promoter element	This study
pBBup14	A pBBR1MCS plasmid containing the 14-kDa protein's gene along with its 5′ flanking sequences that include the putative promoter element	This study
pBBRBS14	A pBBR1MCS plasmid containing the 14-kDa protein's gene along with its 5′ flanking sequences that include the ribosomal binding site but not the putative promoter element	This study
pBBGroERBS14	A pBBGroE plasmid containing the 14-kDa protein's gene along with its 5′ flanking sequences that include the ribosomal binding site but not the putative promoter element	This study
pBB4up14	A pBBR4MCS plasmid containing the 14-kDa protein's gene along with its 5′ flanking sequences that include the putative promoter element	This study

possesses lectin-like properties. Furthermore, deletion of the gene encoding this protein from *B. abortus* 2308 resulted in a rough-like phenotype and a reduction of its virulence in mice.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used for this study are listed in Table 1. The *Brucella* strains were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) plates (Difco Laboratories, Detroit, MI) at 37°C in an atmosphere containing 5% CO_2. Smooth and rough colony phenotypes of *Brucella* strains were determined by crystal violet staining and autoagglutination in 0.1% acriflavin solution per previously described procedures (2). Work with all *Brucella* strains was performed under biosafety level 3 conditions. Killing of *Brucella* strains was accomplished by incubation of the liquid culture at 68°C for 2 h. *Escherichia coli* strains were grown overnight in either yeast-tryptone (2× YT) or Luria-Bertani (LB) broth or on LB agar plates. Bacteria containing plasmids were grown in media containing appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 30 μg/ml.

Amplification of the 14-kDa protein-encoding gene. The different primers used for amplification of the 14-kDa protein-encoding gene along with its upstream region are identified in the context of the open reading frame in Fig. 1. To facilitate directional cloning, a restriction enzyme recognition site was engineered at the 5′ end of each primer, a BglII site was added to all the forward primers (14upstream, 14RBS, and SPlessforw), and a KpnI site was added to the reverse primer (14rev). The reverse primer was used in combination with each of the forward primers to amplify via PCR the respective portions of the open reading frame and the upstream region from the genomic DNA of *B. abortus* 2308. The amplified fragments were cloned into the pCR2.1 vector, using the TA cloning system (Invitrogen, Carlsbad, CA). The nucleotide sequence integrity of the cloned fragments was confirmed by nucleotide sequence analysis.

Overexpression of the 14-kDa protein in *B. abortus* RB51. The 14-kDa protein was overexpressed in *B. abortus* strain RB51 by transformation with either pBBGroERBS14 or pBB14up. The plasmids were electroporated into *B. abortus* strain RB51, and the bacterial colonies containing the plasmids were obtained on a TSA plate containing 30 μg/ml of chloramphenicol. Several colonies of transformed *B. abortus* were grown individually in TSB with chloramphenicol, and bacteria were harvested, heat killed, and used for Western blot analysis.

Expression and purification of recombinant 14-kDa protein from *E. coli*. The 14-kDa protein was expressed in *E. coli* using the expression vector pRSETB (Invitrogen). A DNA fragment encoding the 14-kDa protein without its signal peptide was amplified from *B. abortus* 2308 genomic DNA using primers SPlessfowd and 14rev (Fig. 1). The amplified fragment was cloned into the pCR2.1 vector to generate pTAssL14. After confirmation of the nucleotide sequences, the cloned fragment was excised from pTAssL14, using the BglII and KpnI enzymes, and subcloned into the pRSETB vector to generate pRSETssL14. *E. coli* BL21(DE3) cells were transformed with pRSETssL14, and expression of the recombinant protein was carried out per the manufacturer's recommended procedures. Since the expressed 14-kDa protein was found to be present in an insoluble form, purification of the protein by metal affinity chromatography on ProBond nickel resin was performed under denaturing conditions, using buffers containing 8 M urea, per the manufacturer's instructions (Invitrogen). The purified recombinant 14-kDa protein in phosphate buffer with 8 M urea, pH 4, was used directly in analyses involving sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and red blood cell (RBC) blotting.

The removal of urea through dialysis resulted in precipitation of the protein; various protocols were tried unsuccessfully to remove urea without causing precipitation of the protein. Therefore, for use in hemagglutination and hemagglutination inhibition (HI) assays, the protein was purified using an SP HiTrap ion-exchange column. A 5-ml SP HiTrap column was equilibrated with phosphate-buffered saline (PBS) per the manufacturer's suggestions (Pharmacia Biotech). Lysates of *E. coli* expressing the His–14-kDa protein fusion were prepared by suspending the bacteria in PBS with 6 M urea and sonicating them for 2 min. The clarified *E. coli* lysates were then loaded onto the equilibrated HiTrap column. Following a thorough wash with PBS containing no urea, the bound 14-kDa protein was eluted with 0.5 M NaCl, and 1-ml fractions were collected. Each fraction was analyzed by SDS-PAGE and Western blotting to identify those containing the 14-kDa protein. Fractions containing the 14-kDa protein were pooled and dialyzed against PBS.

Western blotting. Extracts of whole *Brucella* cells, extracts of *E. coli* expressing the 14-kDa fusion protein, and the purified 14-kDa fusion protein were separated in 12.5% or 15% denaturing polyacrylamide gels by electrophoresis per a standard procedure (3). From the gels, the antigens were transferred to nitrocellulose membranes that were subsequently blocked with 3% bovine serum albumin and incubated with various antibodies and appropriate horseradish

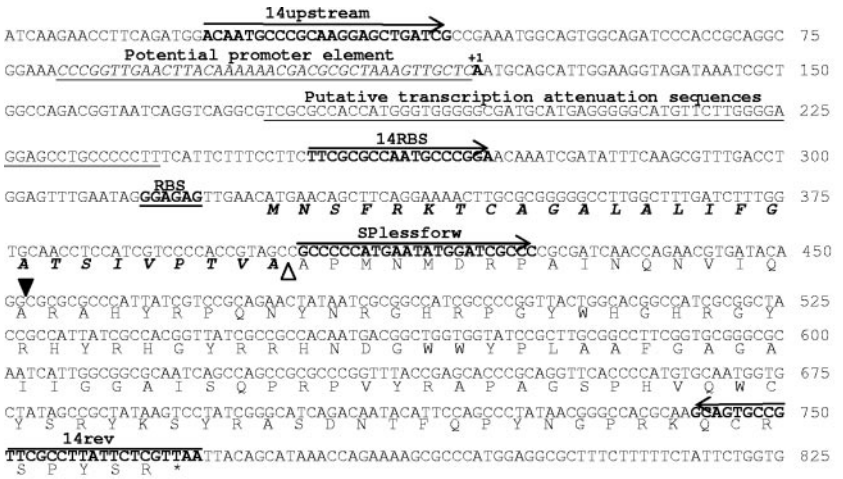

FIG. 1. Nucleotide sequence features and locations of primers within the 14-kDa protein's gene and the 5′ flanking region. Only the top strand of the DNA is shown. The predicted amino acid sequence of the 14-kDa protein is depicted below the gene sequence. Sequences used for synthesizing primers are indicated in bold, and the direction of each primer is shown with an arrow. The ribosomal binding site (RBS), the potential promoter element (identified using an algorithm available at http://www.fruitfly.org/seq_tools/promoter.html), and the putative transcription attenuation sequences (18) are also indicated. The potential transcriptional start site is indicated with "+1." The putative signal sequence is shown in italics, and the predicted signal peptidase cleavage site is indicated with an open triangle. The filled triangle indicates the site of insertion of a chloramphenicol resistance cassette in the disruption mutants.

peroxidase-conjugated secondary antibodies. The membranes were developed with a substrate solution containing 1-chloro-4-naphthol and hydrogen peroxide.

RBC blotting. RBC blotting was performed using a previously published protocol, with some modifications (21). Briefly, blood from various species of animals was collected in tubes containing sodium citrate as an anticoagulant. Erythrocytes were sedimented via low-speed (1,500 rpm) centrifugation and washed four times with 10 volumes of PBS each time. Finally, a 2% erythrocyte suspension was prepared in PBS. Concurrently, protein samples containing the 14-kDa protein were separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 3% bovine serum albumin and then incubated with the 2% erythrocyte suspension for 30 to 60 min at room temperature without any agitation. The membrane was washed gently by dipping it several times into a dish containing PBS. The band exhibiting red color resulting from the interaction of RBCs with the 14-kDa protein was photographed immediately.

Hemagglutination and hemagglutination inhibition assay. Serial dilutions of the purified 14-kDa protein were prepared using PBS. To each well of a U-bottomed 96-well plate (Becton Dickinson Labware, Oxnard, CA), 25 μl of the diluted 14-kDa protein followed by 25 μl of the 2% mouse erythrocyte suspension was added and mixed by gently pipetting up and down. The 96-well plates were incubated at room temperature for 1 h and then visually examined for lattice (positive reaction, i.e., hemagglutination) or button (negative reaction) formation.

In order to determine the affinity of the 14-kDa protein for specific carbohydrate moieties, seven different sugar solutions were tested in a hemagglutination inhibition assay. Stock solutions (1 M) of glucose, galactose, mannose, maltose, methyl-α-D-mannopyranoside, D-galactosamine, and *N*-acetyl-D-glucosamine were prepared in PBS. To each well of a U-bottomed 96-well plate (Becton Dickinson Labware), 25 μl of serially diluted sugar solution was added. Next, 25 μl of the 14-μg/ml protein solution was added and mixed with the sugar solution by pipetting up and down. After incubation of the plate for 60 min at room temperature, 50 μl of 2% erythrocytes was added to each well and mixed by gently pipetting up and down. The plate was then incubated for an additional 60 min at room temperature and examined for button (positive reaction, i.e., hemagglutination inhibition) or lattice (negative reaction, i.e., hemagglutination) formation.

Generation of disruption mutants. A suicide vector, pTAmp14::Cm^r, containing the 14-kDa protein-encoding gene with a disruption, was constructed by subcloning the chloramphenicol cassette from pBBR1MCS into pTAup14 (Table 1 and Fig. 1). *B. abortus* strains 2308 and RB51 were electroporated with pTAmp14::Cm^r, and recombinant colonies that were resistant to chloramphenicol but sensitive to ampicillin were selected for further screening by Southern blotting (22). For Southern blotting, digoxigenin-labeled DNA probes for the 14-kDa protein- and chloramphenicol acetyltransferase-encoding genes were prepared using a DIG DNA labeling and detection kit (Roche Molecular Biochemicals, Indianapolis, Ind.). The genomic DNAs of the 14-kDa protein gene disruption mutants of *B. abortus* 2308 and RB51 and their parental strains were digested with the HindIII enzyme, and the resulting fragments were separated in a 0.8% agarose gel. Southern blotting was performed per the manufacturer's suggested procedures. The confirmed disruption mutants were designated *B. abortus* 2308Δ14 and RB51Δ14.

Complementation of disruption mutant. Complementation of the 14-kDa protein gene mutation in strain 2308Δ14 was accomplished by subcloning the insert of pTAup14 into pBBR4MCS to generate pBB4up14. *B. abortus* strain 2308Δ14 was transformed with pBB4up14, and the colonies containing the plasmid were selected on TSA plates containing ampicillin.

Bacterial clearance experiments with mice. Two separate experiments were performed. For both experiments, the exact dose inoculated into each mouse was determined retrospectively by plating serial dilutions of the bacterial suspensions used. In the first experiment, groups of 15 mice each were inoculated intraperitoneally with 4.6×10^4 CFU of *B. abortus* 2308 and 4.54×10^4 CFU of *B. abortus* 2308Δ14. Five mice from each group were euthanized 1, 7, and 14 days after inoculation, and the bacterial counts in their spleens were determined. In the second experiment, groups of 15 mice each were inoculated with 4.61×10^4 CFU of *B. abortus* 2308, 4.65×10^4 CFU of *B. abortus* 2308Δ14, 5×10^8 CFU of *B. abortus* RB51, and 5.5×10^8 CFU of *B. abortus* RB51Δ14. Five mice from each group were euthanized 14, 28, and 42 days after inoculation, and the bacterial loads in their spleens were determined. Student's *t* test was used to compare the data between the groups inoculated with the wild-type and respective mutant strains.

RESULTS

Overexpression of the 14-kDa protein in *B. abortus* RB51 and *E. coli*. Homologous overexpression of the 14-kDa protein in *B. abortus* strain RB51 was used as a method to obtain preliminary evidence of the functionality of the putative promoter and also to examine the role of this protein's immunological role in protection against brucellosis. *B. abortus* RB51 harboring either pBBGroERBS14 or pBBup14 overexpressed the 14-kDa protein, as shown by Western blotting of the bacterial extracts with goat 48 serum (serum from a goat hyperimmunized with strain RB51 extracts) (Fig. 2). Very low levels of expression of the 14-kDa

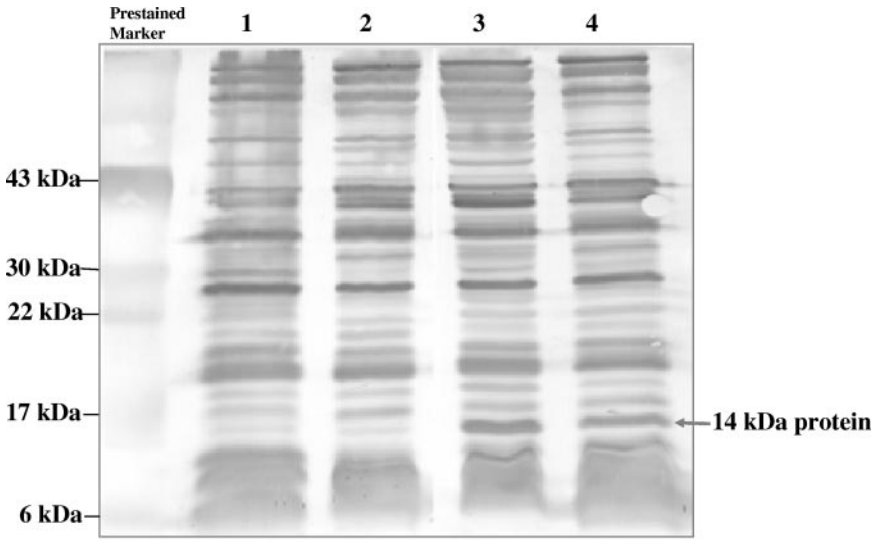

FIG. 2. Overexpression of the 14-kDa protein in *B. abortus* strain RB51. Total antigen extracts of the recombinant RB51 strains were subjected to Western blot analysis using serum from goat 48 as the primary antibody. Lanes 1 to 4 contain the same amount of antigen extracted from strain RB51 containing plasmids pBBR1MCS (lane 1), pBBRBS14 (lane 2), pBBGroERBS14 (lane 3), and pBBup14 (lane 4).

protein were detected in *B. abortus* RB51 and in RB51 harboring either pBBR1MCS or pBBRBS14.

The 14-kDa protein without its putative amino-terminal signal peptide was expressed in *E. coli* with a T7 promoter and a His-tag fusion (~19 kDa) at its amino terminus, and the expressed protein was purified by metal affinity chromatography. In Western blots, the recombinant 14-kDa protein reacted with goat 48 serum and a monoclonal antibody that recognizes a specific epitope (T7 tag) present in the fusion portion of the recombinant 14-kDa protein (Fig. 3A and B). In addition, sera from naïve mice also reacted with the recombinant 14-kDa protein as well as with the 14-kDa protein overexpressed in strain RB51 (Fig. 3C).

Identification of lectin-like properties of the 14-kDa protein. In order to further examine the basis for the observed reaction of naïve mouse serum with the 14-kDa protein, the ability of this protein to bind immunoglobulin G (IgG) from other animal species was examined by Western blot analysis. Antigen extracts of strain RB51/pBBGroERBS14 and the affinity-purified 14-kDa fusion protein were subjected to Western blotting with nonimmune sera from a variety of species, including cattle, goats, rabbits, chickens, mice, rats, and pigs. Appropriate secondary antibodies conjugated with horseradish peroxidase were used to detect the bound IgGs. All the IgGs tested were able to bind to the 14-kDa protein (data not shown). Interestingly, when incubated with secondary antibodies conjugated with horseradish peroxidase alone, the 14-kDa protein failed to bind to these antibody conjugates in Western blots (data not shown). Western blot analysis with Fab and Fc fragments of goat IgG (purchased from Sigma-Aldrich) revealed that the 14-kDa protein bound specifically to the Fc portion but not to the Fab fragment (data not shown). Since immunoglobulins are glycoproteins and their Fc portion contains the carbohydrate moieties, we reasoned that the 14-kDa protein might be actually binding, similar to lectin-like proteins, to some of the carbohydrate moieties present on the IgGs.

To test if the 14-kDa protein has lectin-like properties, RBC blotting was performed, in which a 2% suspension of mouse erythrocytes was used to bind the 14-kDa protein of strain RB51/pBBGroERBS14 or the affinity-purified recombinant fusion protein. Mouse RBCs specifically bound to the 14-kDa protein, as revealed by the presence of a distinct red band corresponding to the appropriate molecular size (Fig. 4). RBC blotting was also performed using erythrocytes from a variety of different species, including humans, pigs, goats, sheep, cows, horses, chickens, rabbits, gerbils, and mice. The 14-kDa protein bound RBCs from mice and gerbils but not RBCs from all other species tested (data not shown).

The lectin-like properties of the 14-kDa protein were further verified by hemagglutination assays. Serial dilutions of the purified fusion protein were prepared in PBS and incubated at room temperature with a 2% suspension of mouse RBCs at a 1:1 ratio (protein to RBC suspension). Wells positive for hemagglutination showed lattice formation, and negative wells showed button formation. The 14-kDa protein was able to hemagglutinate mouse RBCs at a minimum protein concentration of 3.50 μg/ml (Fig. 5).

To determine the specific carbohydrates to which the 14-kDa protein was able to bind, HI assays were performed with seven monosaccharides. Only mannose was able to cause HI at a minimum final concentration of 62.5 mM (Fig. 5).

Construction and characterization of disruption mutants. Electroporation of *B. abortus* strains 2308 and RB51 with the suicide vector pTAmp14::Cm^r resulted in the generation of several hundred chloramphenicol-resistant colonies. Of these, 20 colonies from each strain were subsequently screened on TSA plates supplemented with ampicillin to select for mutants that resulted from the homologous double-crossover recombination event. Seven colonies from each strain were found to be both chloramphenicol resistant and ampicillin sensitive. To ensure that these colonies represented true disruption mutants of the 14-kDa protein-encoding gene, Southern blot analysis

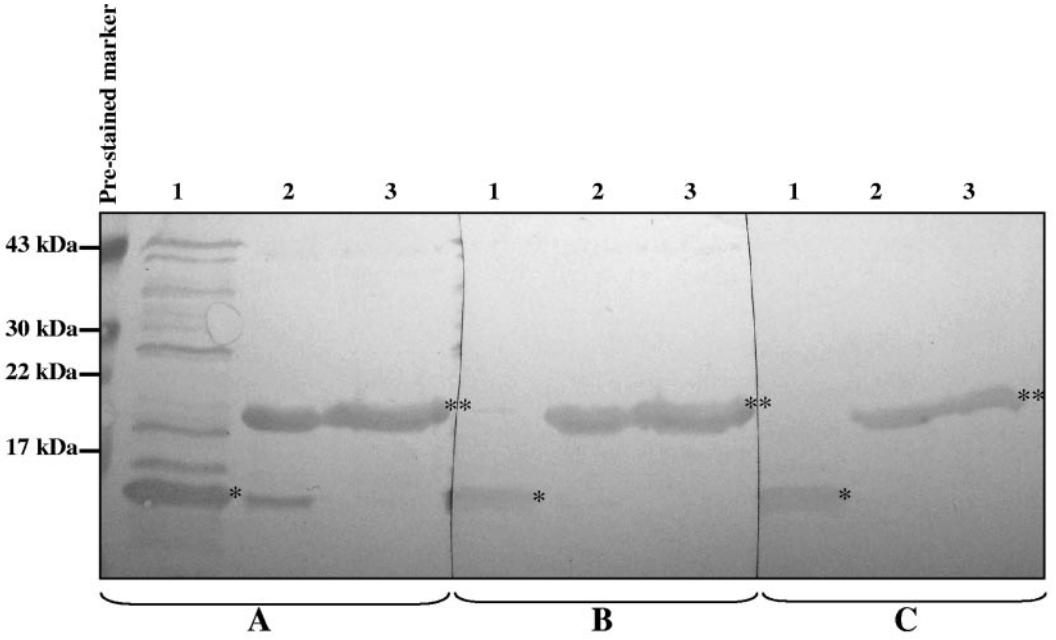

FIG. 3. Western blot detection of the 14-kDa protein's reactivity with specific and nonspecific immunoglobulins. A total antigen extract of RB51 overexpressing the 14-kDa protein under the control of the *groE* promoter (pBBGroERBS14) (lane 1) and the purified recombinant fusion protein of the 14-kDa protein (lanes 2 and 3) were incubated with either goat 48 serum (A), a monoclonal antibody to the T7 tag of the fusion protein (B), or normal mouse sera (C). The 14-kDa protein and its 19-kDa fusion protein are indicated with single and double asterisks, respectively.

was performed with HindIII-digested genomic DNA extracted from a colony from each strain. Digoxigenin-labeled probes prepared with the gene for the 14-kDa protein and chloramphenicol acetyltransferase were used independently to hybridize with the DNA fragments resulting from restriction digestion. The probe specific for the gene for the 14-kDa protein hybridized with an ~4.5-kb fragment of *B. abortus* strain 2308 or strain RB51 and an ~5.6-kb fragment of the two disruption mutants, strains 2308Δ14 and RB51Δ14 (Fig. 6A). The increase in size of the hybridized fragments of the disruption mutants was in accordance with the predicted size indicative of the insertion of the chloramphenicol resistance cassette within the 14-kDa protein-encoding gene. This was further confirmed by hybridization of the chloramphenicol resistance gene-specific probe with the 5.6-kb fragments of the disruption mutants but not with any fragments of strain 2308 (Fig. 6B).

Crystal violet staining revealed that the disruption mutant 2308Δ14 showed a rough-like phenotype, with partial uptake of the dye. When bacterial suspensions of strain 2308Δ14 were mixed with 0.1% acriflavin, autoagglutination, which is an indicator of a rough phenotype, was observed. However, the degree of autoagglutination appeared to be less than that of strain RB51, a known rough strain. No obvious differences were observed between strains RB51 and RB51Δ14 in their

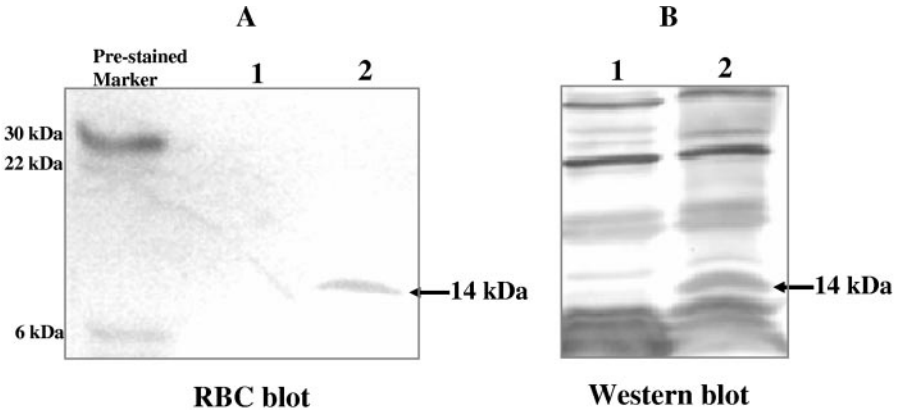

FIG. 4. Demonstration of lectin-like property of the 14-kDa protein by RBC blotting. Total antigen extracts of strains RB51 (lane 1) and RB51/pBBGroERBS14 (lane 2) were separated by 15% SDS-PAGE, transferred to a nitrocellulose membrane, and blocked with 2% bovine serum albumin. Membrane A was incubated with a 2% suspension of mouse erythrocytes as described in Materials and Methods. Membrane B was subjected to Western blot analysis using goat 48 serum as the primary antibody. In both blots, the band corresponding to the 14-kDa protein is indicated.

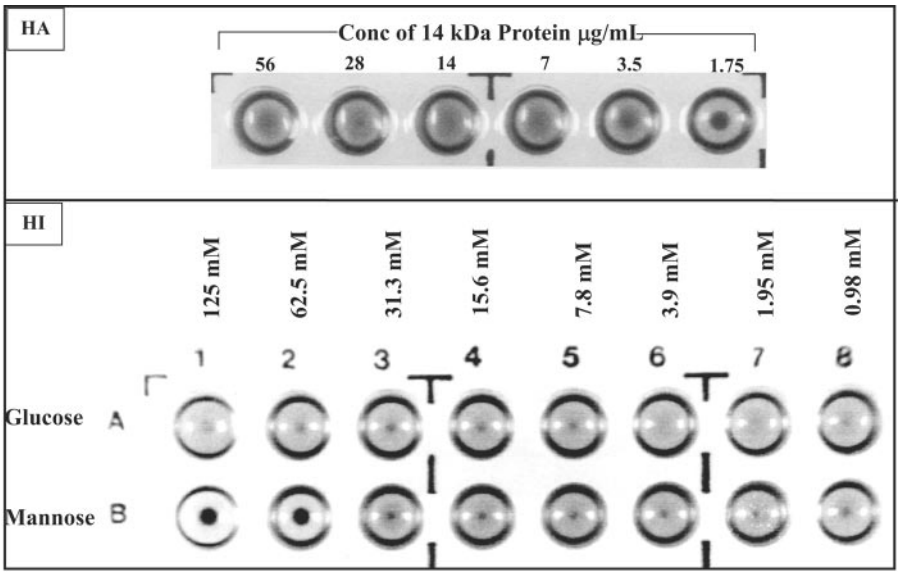

FIG. 5. Hemagglutination (HA) and HI assays with the recombinant 14-kDa protein and mouse erythrocytes. (Top) Serial dilutions of the 14-kDa protein were incubated at room temperature with a 2% suspension of mouse red blood cells. Note the hemagglutination caused by the 14-kDa protein, as demonstrated by lattice formation, up to a dilution containing 3.50 μg/ml of the recombinant 14-kDa protein. (Bottom) The 14-kDa protein was preincubated at 14 μg/ml with serial dilutions of either glucose (row A) or mannose (row B), and 2% mouse RBCs were added. Note the inhibition of hemagglutination, as demonstrated by button formation, by mannose up to a concentration of 62.5 mM.

staining patterns with crystal violet dye and autoagglutination with acriflavin.

To further characterize the rough-like phenotype of the 2308Δ14 disruption mutant, total antigen extracts of the mutants were analyzed by Western blotting with Bru38, a rat monoclonal antibody specific to the *Brucella* O antigen (29). As shown in Fig. 7, strain 2308Δ14 showed an altered O-antigen profile compared with its parent strain, 2308. With strain 2308Δ14, the antibody showed a low degree of reaction with components of 30 to 50 kDa. The Bru38 antibody did not react with antigen extracts of strains RB51 (Fig. 7) and RB51Δ14 (data not shown).

Complementation of strain 2308Δ14 with a functional gene encoding the 14-kDa protein resulted in reversion to the smooth phenotype. The O-antigen profile of the complemented strain was similar to that of parent strain 2308 (Fig. 7).

Persistance of disruption mutant in a mouse model. In order to determine the 14-kDa protein's role in the virulence of *B. abortus*, the 14-kDa protein gene disruption mutants (2308Δ14 and RB51Δ14) were examined for the ability to persist in spleens of BALB/c mice (Fig. 8). The numbers of strain 2308 cells recovered from spleens of infected mice remained 2 to 3 logs higher than those of strain 2308Δ14 cells from day 1 through 42 days postinoculation ($P < 0.001$). Interestingly, there was no significant difference in the numbers of bacteria recovered at different time points during the study from mice inoculated with strain 2308Δ14 ($P > 0.1$). No difference was observed in the numbers of bacteria in the spleens of mice inoculated with either strain RB51 or RB51Δ14, indicating similar attenuation levels ($P > 0.1$).

All bacterial colonies isolated from mice inoculated with the disruption mutants were found to be resistant to chloramphenicol. To verify that these chloramphenicol-resistant colonies represented the disruption mutants, 10 colonies of each mutant were selected at random, their genomic DNAs were extracted, and Southern blot analysis was performed using the digoxigenin-labeled probe for the 14-kDa protein-encoding gene. In every sample, the probe hybridized with a 5.6-kb fragment, indicating the

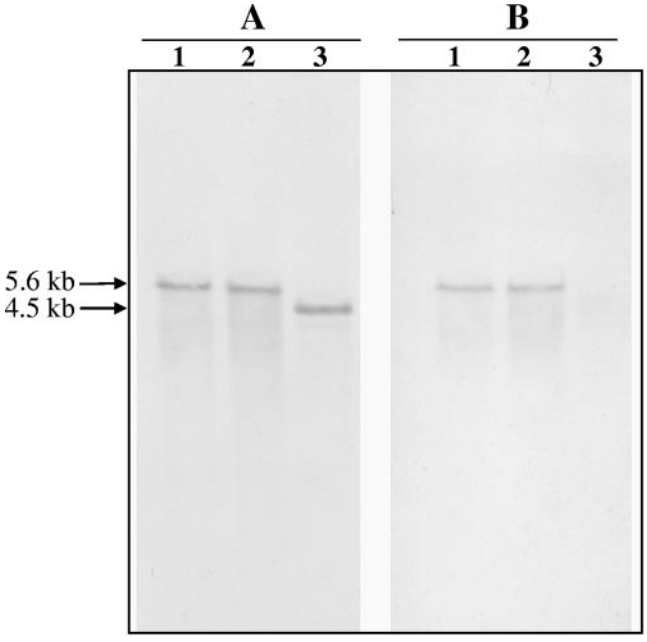

FIG. 6. Southern blot analysis to confirm disruption of the 14-Da protein's gene in *B. abortus* strains 2308 and RB51. Genomic DNAs from strains 2308Δ14 (lane 1), RB51Δ14 (lane 2), and 2308 (lane 3) were digested with the HindIII restriction enzyme and subjected to Southern blot analysis with digoxigenin-labeled probes specific to either the 14-kDa protein's gene (A) or the chloramphenicol resistance gene (B). The approximate sizes of the DNA fragments hybridized with the probes are indicated.

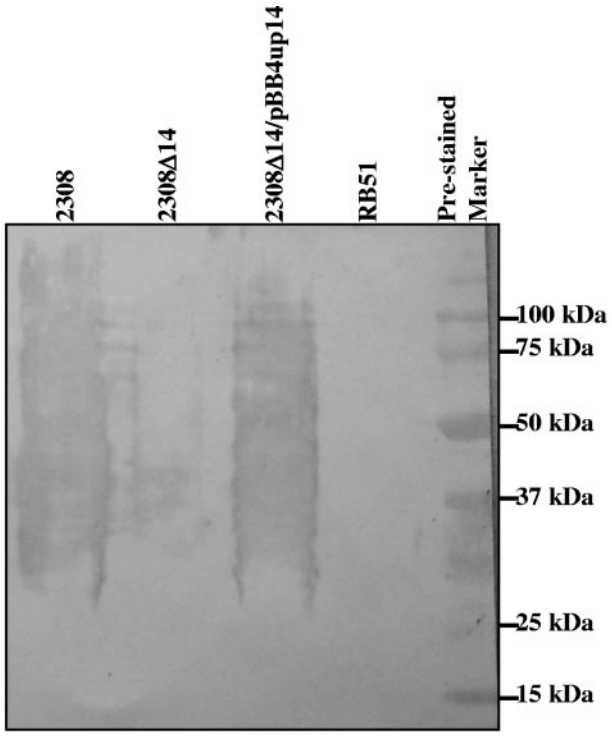

FIG. 7. Western blot depicting the aberrant O-antigen profile of strain 2308Δ14. The same number of whole bacteria of *B. abortus* strains 2308, 2308Δ14, 2308Δ14/pBB4up14, and RB51 were lysed, separated by 12.5% SDS-PAGE, and subjected to Western blot analysis using Bru38, a rat monoclonal antibody against the *Brucella* O antigen.

presence of the disrupted 14-kDa protein-encoding gene. In addition, all of the strain 2308Δ14 colonies recovered from the mouse spleens were found to exhibit a rough-like phenotype based on autoagglutination with 0.1% acriflavin.

DISCUSSION

The 14-kDa protein of *Brucella* was initially identified by Chirhart-Gilleland et al. (6) as an immunoreactive protein to which *Brucella*-infected hosts developed both antibody and T-cell responses (6). In relation to the recently completed full genome sequences of *Brucella* spp., the 14-kDa protein studied here is the product of *B. abortus* gene BruAb2_0497, and its corresponding genes in *B. melitensis* and *B. suis* are BMEII0552 and BRA0735, respectively. Chirhart-Gilleland et al. also first reported the full gene sequence for the 14-kDa protein, and based on upstream nucleotide sequence analysis, they proposed that this gene could be the last gene of an operon (6). However, our search of the upstream nucleotide sequences using the neural network method of promoter prediction available at the Lawrence Berkeley National Library (http://www.fruitfly.org/seq_tools/promoter.html) revealed a potential putative promoter with a high score (0.94) (Fig. 1). Overexpression of the 14-kDa protein in strain RB51 harboring pBBup14, but not pBBRBS14, indicates that the predicted promoter element is functional in *Brucella*. Therefore, it is highly possible that the expression of the 14-kDa protein can occur independently of that of its immediate upstream genes, although further studies are need to determine the true promoter(s) involved in the expression of the 14-kDa protein. A comparison of the Western blot profiles of strain RB51 and overexpressing recombinant strains (RB51/pBBup14 and RB51/pBBGroE/RBS14) revealed that the level of expression of the 14-kDa protein in strain RB51 is low. A similar observation was also made with *B. abortus* strain 2308 (unpublished observation). The low level of 14-kDa protein expression in *B. abortus* could not be solely because of the single copy of the gene present on the chromosomal DNA, since higher levels of expression of other proteins from single-copy genes have been observed in *Brucella* (27). It is possible that the promoter of this gene is inherently weak, leading to less expression of the protein when the gene is present as a single copy. It is also possible that the expression of this protein in *Brucella* is regulated so that only minimal expression is seen when the bacteria are grown under in vitro culture conditions. Interestingly, recently reported computational analyses identified nucleotide sequence elements between the promoter and the ribosomal binding site of the 14-kDa protein's gene that are potentially involved in transcription-attenuation mechanisms (Fig. 1) (18; *Brucella*-specific information can be accessed at http://cmgm.stanford.edu/~merino/). This finding suggests that the expression of the 14-kDa protein is potentially regulated at the level of transcription.

Our Western blot analyses indicated that the 14-kDa protein could bind, in a nonimmune fashion, with IgGs from several animal species, most likely through interaction with the Fc portion of the antibody. This can be interpreted as an immunoglobulin binding property of this protein. However, the abilities of the 14-kDa protein to interact with and agglutinate erythrocytes in RBC blotting and hemagglutination tests, respectively, suggest that this protein actually possesses lectin-like properties. Therefore, the interaction of the 14-kDa protein with IgG could be through its affinity for the carbohydrate moieties present in the Fc portion, although we cannot rule out the possibility that the 14-kDa protein could have both lectin-like and immunoglobulin binding properties. Of the seven sugars tested in HI experiments, only mannose inhibited the agglutination reaction, suggesting that the 14-kDa protein can bind with this sugar in vivo. However, further studies with other monosaccharides, disaccharides, and other carbohydrate moieties have to be performed to determine if the 14-kDa protein can bind with other sugar molecules.

The functional significance of the observed immunoglobulin binding and hemagglutination properties of the 14-kDa protein in *Brucella* infection remains to be examined. It is well documented that immunoglobulin binding proteins and hemagglutinins of some bacteria play an essential role in establishing infection and/or causing pathogenesis (5, 12, 20, 25). Nonetheless, it is likely that our in vitro observations merely reflect the lectin-binding properties of the 14-kDa protein, and the in vivo function of this protein may be unrelated to its immunoglobulin binding and hemagglutination features. Recently, del C Rocha-Gracia et al. (8) reported that *Brucella* spp. can cause hemagglutination through binding with sialic acid residues present on erythrocytes. Furthermore, they identified a 29-kDa heat-extractable *Brucella* surface protein that bound to erythrocytes through interaction with surface-exposed sialic acid residues; the 29-kDa protein was identified to be the product

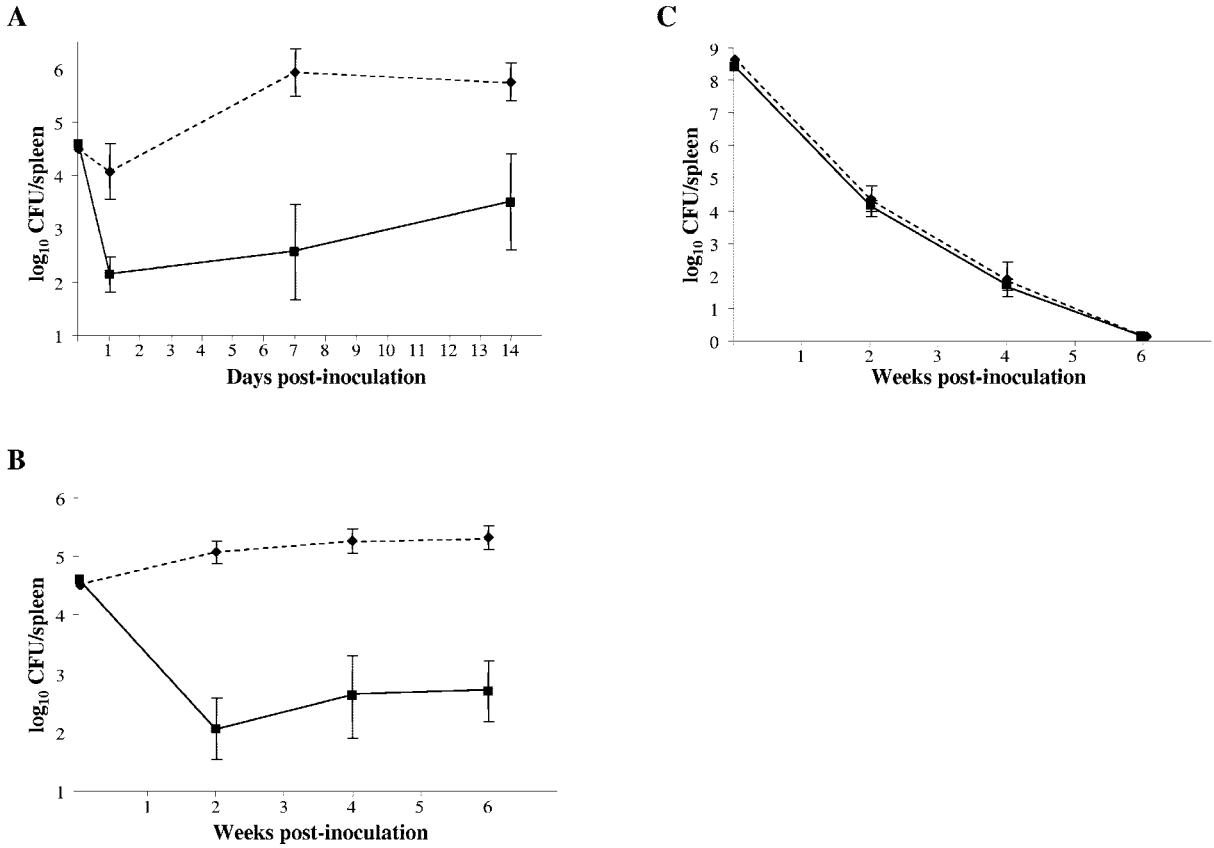

FIG. 8. Persistence of *B. abortus* strains in mice. (A and B) Groups of mice were inoculated intraperitoneally with strains 2308 (dashed lines) and 2308Δ14 (solid lines), and on days 1, 7, and 14 (A) or weeks 2, 4, and 6 (B), five mice from each group were euthanized, and the bacterial burdens in their spleens were determined. (C) Groups of mice were inoculated intraperitoneally with strains RB51 (dashed lines) and RB51Δ14 (solid lines), and at weeks 2, 4, and 6, five mice from each group were euthanized, and the bacterial burdens in their spleens were determined. The results shown are mean $\log_{10}$ CFU/spleen ± standard deviations.

of the *B. melitensis* gene BMEII0435, which encodes a putative ribose-binding periplasmic protein (8).

Disruption of the 14-kDa protein-encoding gene in strain 2308 unexpectedly resulted in a rough-like phenotype, and the mutant strain contained an aberrant O-antigen profile in Western blots. Complementation of the mutant strain with pBB4up14 restored the smooth phenotype, indicating that the observed rough-like phenotype of the mutant strain was indeed because of the absence of the 14-kDa protein. The actual role that this protein plays in the biogenesis of smooth lipopolysaccharide of *Brucella* remains to be determined. In mouse infection studies, the mutant strain 2308Δ14 exhibited a reduced ability to colonize mouse spleens at all time points tested, starting at day 1 up until 6 weeks postinfection. The ability of the complemented mutant strain (2308Δ14 with pBBup14) to replicate in mouse spleens was tested 1 and 2 weeks after inoculation and was found to be similar to that of the parent strain (unpublished data). Interestingly, the CFU of strain 2308dΔ14 in mouse spleens remained at the same level (~2.5 log) throughout the duration of the study. Conversely, disruption of the 14-kDa protein-encoding gene had no effect on the clearance pattern of strain RB51, a rough strain, from mouse spleens. However, since *B. abortus* RB51 is an attenuated strain, it is possible that the 14-kDa protein gene disruption had little or no effect on its persistence in mouse spleens. Nevertheless, based on the rapid decrease in bacterial numbers on day 1 postinfection, it appears that the reduced virulence of strain 2308Δ14 is most likely because of its rough-like phenotypic characteristic. For *B. abortus*, it is a well-known fact that rough mutants are less virulent than their smooth parent strains. However, previous reports by other researchers indicated that not all rough mutants show the same level of attenuation (1, 17, 19, 26). The ability of strain 2308Δ14 to survive in mouse spleens for up to 6 weeks postinfection without any significant change in the bacterial number suggests that although it is unable to replicate to higher numbers, this mutant strain is capable of establishing a persistent infection similar to that of its parent strain, but with a low bacterial burden. This feature, to the best of our knowledge, has not been observed with other *B. abortus* rough mutants; all of the rough mutants studied so far are cleared from mice gradually, with the actual time taken for complete clearance depending on the initial inoculation dose and the nature of the mutation.

Although the findings presented in this paper reveal an essential role for the 14-kDa protein in *B. abortus* virulence, the specific function(s) of this protein in *Brucella* remains to be elucidated. Based on computer-aided analysis of the deduced amino acid sequence, this protein is a highly basic (pI 11.47)

protein with a consensus signal peptide at the amino terminus and a membrane-spanning domain in the middle. Therefore, this protein may potentially be located in the inner or outer membrane of *Brucella* and may be involved in sugar or other polysaccharide transport mechanisms. The gene for the 14-kDa protein is present in all six *Brucella* species (6; our unpublished data). Based on recently completed genome sequences, the 14-kDa protein's gene and amino acid sequences in *B. melitensis*, *B. suis*, and *B. abortus* are identical. BLAST searches of known databases indicate that homologs of the 14-kDa protein are also present in *Agrobacterium*, *Sinorhizobium*, *Mesorhizobium*, and *Bartonella*, which are phylogenetically closely related to *Brucella* (data not shown). In addition, two (BMEI0627 and BMEI 1760 of *B. melitensis*) or three (BR1377, BRA0587, and BR0187 of *B. suis*; BruAb2_0637, BruAb1_0183, and BruAb1_1373 of *B. abortus*) other *Brucella* genes encode hypothetical proteins that show significant sequence similarity with the 14-kDa protein; the sequence identity is especially high for the 40 to 50 amino acids of the carboxy-terminal portion of the 14-kDa protein (data not shown). It would be interesting to know if these gene products and the 14-kDa protein participate in mechanisms involving similar functions.

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